HIGH EXPRESSION AND PRODUCTION OF HIGH-SPECIFIC ACTIVITY RECOMBINANT S-ADENOSYL HOMOCYSTEINASE (SAHH) AND IMPROVED ASSAYS FOR S-ADENOSYLMETHIONINE (SAM)

This application claims priority under 35 United States Code § 119(e) from provisional application serial No. 60/176,444 filed 14 January 2000, the contents of which are incorporated herein by reference.

Technical Field

The invention relates to a novel recombinant S-adenosyl homocysteinase (SAHH) and methods of using such SAHH. The invention is also directed to diagnostic methods to monitor subjects who have been administered S-adenosylmethionine (SAM), using SAHH. The improved methods of the invention provide rapid and accurate assessment of the concentrations of SAM.

Background Art

The administration of S-adenosylmethionine (SAM) as a "nutraceutical" or as a prescribed medication has recently been suggested as an antidepressant, a preventative or therapeutic component in ameliorating liver disease, and a means to diminish the symptoms of arthritis. The mechanism whereby SAM is believed to act is not understood completely, but it is believed that the relative concentrations of SAM and homocysteine, which is a metabolic product of SAM, affect methylation levels which, in turn, have profound physiological effects. In view of the importance of this drug, it would be desirable to have a reliable and easily performed method to monitor the concentration of the administered pharmaceutical. The present invention provides an improved method to assess therapeutic levels of SAM in subjects administered this drug using S-adenosyl homocysteinase (SAHH). The present invention is also directed to a recombinantly produced SAHH that differs from the previously reported SAHH.

S-adenosylhomocysteinase (S-adenosylhomocysteine hydrolase; SAHH, EC 3.3.1.1) catalyses the reversible conversion of SAH to homocysteine and adenosine (de la Haba and Cantoni, 1959). Various structural analogues of adenosine inactivate SAHH from a number of organisms, resulting in cytotoxicity (Ueland, 1982). Inhibition of SAHH activity by the nucleoside analogues depends on the inhibitor structure as well as the source of the enzyme. SAHH was initially cloned from the *Trichomonas vaginalis* gene and previously characterized (Bagnara *et al.*, 1996).

Minotto, L., Ko, G.-A., Edwards, M. R., and Bagnara, A. S. [*Trichomonas vaginalis E*xpression and characterization of recombinant S-adenosylhomocysteinase. *Experimental Parasitology* 90, 175-180, 1998] have further characterized the *T. vaginalis* SAHH. The gene encoding S-adenosylhomocysteinase in *Trichomonas vaginalis* was expressed on pQE-30 in *Escherichia coli* to facilitate the characterization of the enzyme. A 6×His N-terminal tag expression system (QIAGEN) enabled one-step purification of six mg of rSAHH, obtained from a 100-ml bacterial culture by affinity chromatography using a nickel-NTA matrix. The recombinant enzyme was found to have molecular weight of approximately 56,000. Properties of rSAHH include a similar apparent K_m for adenosine of 20-25 μM for the recombinant and similar inhibition/inactivation patterns adenosine analogues such as arabinosyl adenine (ara-A).

The results of Minotto *et al.*, 1998, differ from the finding of others who have shown that the hydrolase can exist in various oligomeric forms depending on the source of the enzyme. The SAHH activity from prokaryotes is as a hexamer (Shimizu *et al.*, 1984) or tetramer (Porcelli *et al.*, *Biochim. et Biophys. Acta*, **1164**, 179-188, 1993). The enzyme from rat liver (Fujioka and Takata, *J Biol. Chem.*, **256**, 1631-1635, 1981), calf liver (Richards *et al.*, *J. Biol. Chem.*, **253**, 4476-4480, 1978), and other animal sources (Doskeland and Ueland, *Biochem et Biophys Acta* **708**, 185-193, 1982) is tetrameric but with uncertainty whether the subunits are identical or similar. SAHH from a plant source is functional as a homodimer (Guranowski and Pawelkiewicz, *Eur. J. Biochem.* 80, 517-

523, 1977). This is the first report of an SAHH activity being functional in the monomeric form (Minotto *et al.*, 1998).

Disclosure of the Invention

The present invention relates to an improved and novel method for analyzing SAM levels in a sample. In one aspect of the invention, this method may be used to assay therapeutic levels of SAM in a sample from a subject such as, but not limited to, a patient being administered this compound. The method may also be used, to assay SAM levels in a biological fluid such as, but not limited to, blood or other biological fluids of a subject. Such methods may be conducted *in vivo*, such as in the bloodstream, or *in vitro*, such as with a sample taken from a subject. The methods may be used as part of a diagnostic protocol or as part of a therapeutic protocol. As part of a therapeutic protocol, the methods may serve in part to monitor the conditions or progress of the therapy.

In one embodiment of the invention, the assay method may be performed by contacting a sample with glycine N-methyltransferase (GMT), glycine, and SAHH activity. Determination of SAM levels in the sample may then be made by measuring one or more reaction products in the sample, wherein the amount of reaction product(s) is directly proportional to SAM levels in the sample. In one embodiment of the invention, the reaction product homocysteine (HC) is measured directly or indirectly. Indirect measurements of HC may be made by any means including, but not limited to, treatment with homocysteinase (HCYase) and measuring the levels of one or more reaction products (e.g. alpha keto glutarate, H_2S, or NH_3). The H_2S reaction product may be measured directly or indirectly by measuring absorbance or fluorescence. One means of measuring fluorescence is by use of a fluorescence generating reagent.

The invention also provides a novel SAHH, nucleic acids that encode it, compositions comprising it, and methods for its preparation and use. The SAHH contains an amino acid sequence encoded by SEQ ID NO:1. Nucleic acids which encode the SAHH of the invention may be placed in any appropriate nucleic acid vector for

propagation, amplification or expression. The nucleic acids may also be operably linked to other nucleic acids to permit the expression of the SAHH covalently linked to one or more additional amino acids. The additional amino acids result in the production of a hybrid or chimeric protein comprising SAHH. In one preferred embodiment of the invention, the additional amino acids are those of a histidine tag (His tag) which improves subsequent purification of the SAHH of the invention.

The nucleic acids of the invention may be introduced into any appropriate host cell or organism, such as, but not limited to, bacteria, fungi, and higher eukaryotic cells. These cells may be used to recombinantly express the nucleic acids of the invention, optionally followed by isolation and/or purification of the expressed protein. Alternatively, the nucleic acids may also be expressed by use of *in vitro* expression systems.

Purification of the SAHH of the invention may be by any convenient or appropriate means such as, but not limited to, precipitation and/or chromatography. In a preferred embodiment of the invention, the purification is performed in whole or in part by affinity chromatography based on interaction with a His tag. In another preferred embodiment of the invention, the SAHH is purified such that it appears as a single band when analyzed by SDS polyacrylamide gel electrophoresis.

The SAHH of the invention may also be formulated into compositions, such as those comprising pharmaceutical agents or excipients. The SAHH may also be used in the methods of the invention, such as the assay methods described above, as well as additional methods such as that for assaying homocysteine to SAH conversion in a sample to measure homocysteine levels. In another aspect of the invention, the SAHH may be used in methods of depleting excess homocysteine in a sample *in vivo* or *in vitro* by conversion to SAH. Of course the samples of the invention may be any biological fluid of interest.

Brief Description of the Drawings

Figure 1 depicts the pTrcSAHH as inserted to pTrc multiclone site NcoI and BamHI.

Figure 2 contains the results of a stability study of SAHH.

Figure 3 shows screening clones of SAHH.

Figure 4 depicts the pTrcHis·SAHH as inserted to pTrc multicloning site NcoI and BamHI.

Figure 5 contains the results of a stability study of His·SAHH.

Figures 6a-c is an alignment of the nucleotide sequence of SAHH of the invention with the wild type sequence.

Modes of Carrying Out the Invention

The invention provides an isolated and recombinant nucleic acid encoding SAHH comprising SEQ ID NO:1, as well as the corresponding SAHH amino acid sequence. In another aspect, the SAHH gene is modified to encode a modified His•SAHH, which has an extra six histidines, in the N-terminal of the SAHH gene.

In another aspect of the invention, the invention provides methods for the propagation and maintenance of the nucleic acids and their use in the expression of SAHH proteins. The invention further provides methods for the purification of SAHH by single or two step purification methods.

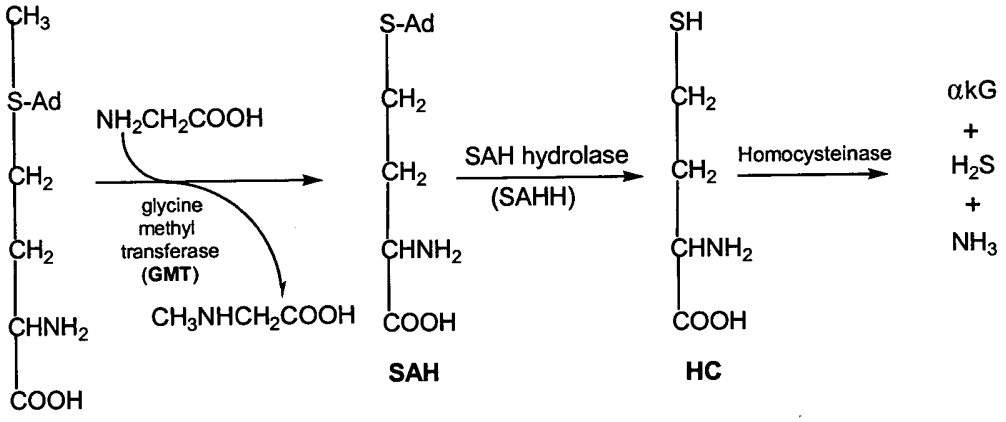
The invention is directed, in one embodiment, to the measurement of SAM in biological fluids. As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, blood and blood fractions, plasma, serum, cerebral spinal fluid, lymph fluid, urine, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, body secretions, tears, saliva, milk, lymphatic or other extracts taken from an animal, cells (including but not limited to blood cells), tumors, organs, and also samples of in vitro cell culture constituents. Measurement in plasma or serum is preferred.

As used herein, "expression" includes transcription and/or translation.

As used herein, the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

SAHH is the enzyme responsible for the conversion to homocysteine from S-adenosyl homocysteine (SAH), which ultimately lowers the level of SAM. Since the levels of SAM administered for therapeutic purposes are very high in proportion to endogenous levels either of SAH or homocysteine (HC), the following scheme can be used to assay for SAM levels in subjects being administered this compound. This assay thus serves as a drug monitoring device, which can be in the form of a kit. The outline of the assay is shown in the scheme below.



As described herein, this general approach to assaying SAM is improved by efficient production of SAHH or His•SAHH and by selection of a homocysteinase which has a high specificity for homocysteine. Although the levels of homocysteine are small relative to those of SAM, thus assuring that the estimate of SAM is not appreciatively distorted by endogenous SAH or HC, the presence of cysteine in bodily fluids at levels significantly higher than the levels of the homocysteine may result in interference if an

enzyme of insufficient specificity is used. The end-product measured in the method of the invention is hydrogen sulfide in the presence of a fluorescence-generating reagent.

The SAHH also catalyzes the reverse reaction of the conversion of homocysteine to SAH, ultimately elevating the level of SAM. Such a reaction is useful in another type of assay, an enzyme-conversion immunoassay of homocysteine, in which homocysteine is measured. Specifically, the SAHH or His•SAHH of the invention is used to quantitatively convert homocysteine to SAH and then the end-product, SAH, is then measured using a standard ELISA assay. This can be performed by providing a sufficient or higher (even excess) amount of SAH. Alternatively, a fluorescent antibody to SAH can be used for quantitation of the resulting SAH. Such an immunoassay can be used as a kit and can be useful for measuring plasma homocysteine, for example, in a range of approximately 1-100 μM.

SAHH or His•SAHH of the invention can also be used as a reagent, in particular for screening for inhibitors and inactivators of the enzyme for use as reagents themselves and as potential therapeutics, for example, in cancer, malaria, arthritis, and other diseases. The SAHH reagent preferably is in the form of a kit that contains an assay, which is simple due to coupling with homocysteine and measurement of the resulting hydrogen sulfide with a dialkyl phenylene diamine reagent such as DBPDA.

Other uses of recombinant SAHH include a therapeutic cancer gene for combination with SAH analogs, which would act as enzyme activated prodrugs with toxicity provided by toxic adenosine analogs released by SAHH. Such adenosine analogs would not be toxic when conjugated to Hcy as an SAH analog. Analogs of homocysteine could also be used, such as selenohomocysteine conjugated with adenosine or an adenosine analog, which in combination with SAHH and rMETase gene therapy would release the very toxic hydrogen selenol as well as the toxic adenosine analog in cancer cells transduced with the two genes.

A preferred embodiment includes a kit for assaying a sample. Preferably, a kit contains instructions for performing the assay, which instructions may be printed on a

package insert, packaging or label included in the kit. The printed matter can also be included on receptacles included in the kit, and indicia of sample and reagent volumes can be indicated in the test receptacle. The precise instructions would vary depending upon the substance to be detected and/or detection method used, but may include instructions for one or more of the following: instructions for dilution of the kit components and/or the sample if necessary, directions for volume or concentration of enzyme used for each assay, volume of sample to add to the assay, directions for adding fluorescence-generating reagents, directions for taking measurement of products, preferred temperature conditions, and timing of component addition and mixing, and use of a standard to calibrate test results.

Production of the SAHH of the invention may be performed by any conventional means. By way of example, and without limiting the scope of the invention, an appropriate vector encoding a SAHH of the invention may be used to first transform bacteria used to express the enzyme. The transformed bacteria can then be cultivated (fermented) in liquid culture for a number of hours until they reach a high density. If the SAHH encoding sequence is under the control of an inducible promoter, the appropriate inducer may be added. After cultivation, the cells may then be harvested by centrifugation and stored frozen until used.

Frozen cells may be thawed and lysed prior to the addition of components to precipitate cell debris. The debris may be collected by centrifugation and the supernatant containing SAHH activity collected. The supernatant can be diluted with an appropriate buffer prior to loading on a prepared chromatographic column. The SAHH may be eluted by a gradient, or more preferably by single step elution in a small volume. The SAHH can then be formulated into a storage preparation prior to use.

In addition to the single step purification protocol provided above, His tag containing SAHH may be purified by affinity chromatography. By way of example and without limiting the invention, bacterial cells expressing His tag SAHH may be cultivated and harvested as described above. The frozen cells may then be thawed and disrupted as

described above to prepare a cell suspension. Solid ammonium sulfate may then be added to the suspension and the mixture kept on ice followed by centrifugation. The supernatant containing SAHH activity is then collected and applied to a previously prepared (equilibrated) Ni – NAT chromatography media. The column is then washed and then developed with a single step elution. Active fractions may be pooled and dialyzed prior to formulation into conditions for frozen storage.

As illustrated in the following examples, SAHH encoding sequences were cloned from *Trichomonas vaginalis* and expressed in *E. coli* according to the procedures described below. The nucleotide sequence for the gene encoding SAHH is provided herein, along with a comparison of the wild type sequence, which is equivalent to the sequence disclosed in Minotto *et al.,* 1998, as shown in Figures 6a-c. A comparison of the two sequences reveals eleven point mutations, which are listed below in Table 1.

TABLE I

COMPARISON OF SAHH SEQUENCES

There are 11 point mutations.

	Wild type	A/C's	Change of amino acid
No. 19	(G)CT	(A)CT	Ala. → Thr.
No. 201	GC(G)	GC(C)	
No. 207	CT(T)	CT(C)	
No. 210	AT(T)	AT(C)	
No. 501	GT(C)	GT(T)	
No. 744	GT(G)	TG(C)	
No. 834	GG(G)	GG(C)	
No. 897	CC(T)	CC(A)	
No. 917	G(T)C	G(C)C	Val. → Ala.
No. 1314	GA(T)	GA(A)	Asp. → Glu.
No. 1346	G(T)T	G(C)T	Val. → Ala.

The SAHH enzyme of the invention has favorable properties. For instance, the SAHH has a high specific activity of at least 1.5 U/ml. Further, the cloned SAHH of the invention provides a high expression of 20% of total cell protein. Moreover, SAHH produced according to the method of the invention has high stability as illustrated in the attached Figures 2 and 5. For example, SAHH and SAHH·His is stable at 45° for three days without activity loss as illustrated in Figures 2 and 5 respectively.

The following example is intended to illustrate but not to limit the invention.

Example 1

Cloning of the SAHH Gene into the pQE-30 Expression Vector

The genomic sequence encoding SAHH in *T. vaginalis* (Bagnara *et al.*, 1996) was amplified by PCR using oligonucleotide primers containing engineered restriction enzyme sites for *BamHI* and *Pst*1 in the upstream (sense) and downstream (antisense) primers, respectively (restriction sites are underlined in both cases): upstream primer, 5′TTTTGGATCCGCTTGCAAATCACCTGCTGGTGC 3′; downstream primer, 3′ CTGCTATCGAGGGGGACGTCTTTT 5′. The recombinant expression vector pQE-30 was transformed into the *Escherichia coli* host strain M15[pREP4] (Villarejo and Zabin, 1974) (QIAGEN).

Example 2

Expression, Purification, and Analysis of Recombinant SAHH

Clones containing the pQE-30-SAHH construct were grown overnight in *E. coli* M15 with ampicillin (100 μg/ml) and kanamycin (25 μg/ml). Expression was induced with 0.1 mM IPTG, followed by growth at 39° C for 14 h with vigorous shaking. Harvested cells were disrupted by sonication in 50 mM Na-phosphate, pH 8.0, 300 mM NaCl followed by centrifugation at 12,000g for 20 min. The recombinant enzyme was then isolated by differential elution from the Ni-NTA column with 50 mM Na-phosphate,

pH 6.0, 300 mM NaCl, 10% glycerol containing various concentrations of imidazole. Aliquots of the purified recombinant enzyme were stored at 4° C without additional glycerol) while other aliquots were mixed with glycerol (50% final concentration) for storage at -20 and -79° C to determine the effect of storage on enzyme activity. The size of the active recombinant enzyme was also analyzed under nondenaturing conditions using size-exclusion capillary chromatography (Superdex 200 PC column) on a Pharmacia Biotech SMART chromatography system.

Example 3

Expression of SAHH in E. coli

The expression of the SAHH gene has been achieved in *E. coli*, a host which provides an SAHH-negative background (Shimizu *et al.*, *Eur. J. Biochem.*, **141**, 385-392, 1984). The *E. coli* clones containing the recombinant SAHH gene sequence exhibited a high degree of expression of the enzyme but largely as insoluble "inclusion bodies" when induced at 37° C with 1-2 mM IPTG. Lowering the temperature to 30° C and decreasing the concentration of IPTG to 0.1 mM decreased the level of expression and resulted in a greater proportion of the enzyme being expressed in a soluble and active form. The recombinant SAHH comprised approximately 12% of the total soluble protein.

Example 4

Purification and characterization of the Recombinant Protein

The recombinant SAHH was purified by affinity chromatography on an NI-NTA column. The molecular weight of the enzyme was an apparent 55,000-56,000 (Fig. 1). The results of the size-exclusion chromatography using a Superdex 200 PC capillary column indicated that the molecular weight of the recombinant enzyme was about 55,000 under nondenaturing conditions. The enzyme is active under these conditions and that SDS-PAGE demonstrated subunit molecular weight of approximately 55,000-56,000, these data indicate that the *T. vaginalis* enzyme is functional as the monomer. This result

differs from the findings of others who have shown that the hydrolase can exist in oligomeric form, with the quaternary structure depending on the source of the enzyme.

Example 5

Fermentation and Purification of S-adenosyl-L-homocysteine Hydrolase

Fermentation:

1. 10 µl of bacteria from mast cell bank were inoculated to 5 ml L.B. and cultivated with shaking at 37° C for 6 hours.
2. 0.5 ml of bacteria from step 1 were transferred to 3 bottles of 400 ml L.B. and cultivated with shaking at 37° C overnight.
3. The cells were collected by centrifuging at 3000 rpm at 4° C, suspended in L.B., and seeded to ferment.
4. Cells were cultivated at fermentor for approximately 6 hours at 28° C until cell density reaches OD600 7.
5. The cells were induced by adding 0.1 mM IPTG and cultivated at 28° C overnight.
6. The cells were harvested by centrifuging at 4000 rpm at 4° C and stored at -80° C until purification.

Purification:

1. The cells were lysed by pass through the homogenizer three times.
2. The cell lysis was mixed with 2% PEI, 30% alcohol, and 8% PEG and heated in waterbath until temperature reached 37° C.
3. Cell debris was discarded by centrifuging at 15,000 rpm for 30 minutes and the supernatant was collected.
4. The supernatant was diluted two-fold by adding 20 mM potassium phosphate buffer pH 8.3, 1mM DTT and EDTA.
5. The supernatant was loaded to pre-equilibrium DEAE-Sepharose fast flow column.

6. The column was prewashed with 20 mM potassium phosphate buffer pH 7.6, 40 mM NaCl,
1 mM DTT and 1 mM EDTA until OD280 reached less than 0.2.
7. SAHH was eluted by 20 mM potassium phosphate buffer pH 7.6, 100 mM NaCl, 1 mM DTT and EDTA.
8. Final product was formulated by adding 30% glycerol and 1mM NAD to the SAHH elution.

Specificity

High expression clone expressed SAHH 20% of total protein. After purification, the specific activity of SAHH was 1.64 units/mg protein. The purity reached over 90%.

Stability

The enzyme was formulated as follows: 20 mM potassium phosphate buffer, pH 7.6, 100 mM sodium chloride, 30% glycerol, 1 mM DTT, 1 mM NAD, and 1 mM EDTA. See Figure 2.

Example 6

SAHH Activity Measurement

Reagent:

Assay buffer: 20 mM potassium phosphate, pH 8.0, 1 mM DTT, and 1 mM EDTA.
2 mM S-adenosyl-L-homocysteine (SAH)
rHCYase (5mg/ml)
L-homocysteine (various concentrations)
40 mM DBPDA, dissolved in 6 M HCl
40 mM potassium ferricyanide

Assay procedure:

	Blank	Standard curve	Test

Assay buffer (μl)	940	970	920
rHCYase (μl)	10	10	10
L-homocysteine (μl)	---	20	---
SAH (μl)	50	---	50
Sample (μl)	---	---	20

Mix well and incubate at 37° C for 5 min.

DBPDA (μl)	50	50	50
Potassium ferricyanide (μl)	50	50	50

Mix well and incubate at 37° C for 10 min. and read absorbance at 675 nm or fluorescence at EX665nm/EM690nm. 1 unit is defined as 1 μmole of S-adenosylhomocysteine hydrolated in 1 min. at 37° C in the presence of excess rHCYase.

Example 7

rSAHH•His Tag Preparation

To construct the expression vector, the SAHH gene was modified by PCR. The 5' primer is CATCATCATCATCATCACGCTTGCAAATCACCTACTGG

6 x His•Tag

and the 3' primer is ATGCATGGATCCTTAATAACGGTAAGCATC.

BamH I

The pTrc 99A(Pharmacia Biotech) was employed as a expression vector. The modified His•SAHH which has extra six histidine codes in N-terminal of SAHH gene was inserted into Nco I-blunt and BamH I site. *E. coli* JM109 was employed as the host strain for His•SAHH expression.

Example 8

Purification of Recombinant S – Adenosylhomocysteine Hydrolase with His-tag

Cell Disruption:

500 grams of frozen cells (-80° C) of E. coli in which SAHH was expressed were thawed and suspension in 500 ml 20 mM potassium phosphate buffer pH 7.6 containing 1 mM DTT and 1 mM EDTA. Disruption of cells with homogenizer (HC-8000, Microfluidics International Corporation) at 5,000 psi for three times.

Ammonium Sulfate Precipitation:

Crystalline ammonium sulfate (20% w/v) was added to disrupted cell suspension. After mixture on ice for 20 minutes, the preparation was centrifuged at 12,000 rpm, 4° C for 30 minutes, then collected the supernatant for further purification.

Ni-NAT Superflow Chromatography:

The clear supernatant containing 10 mM imidazole was applied to Ni – NAT Superflow column (2.0 x 20 cm) equilibrated with Binding Buffer (50 mM potassium phosphate pH 7.6, 0.5 M NaCl, 10 mM imidazole, 1 mM EDTA and 0.01% α-mercaptoethanol). The column was washed with 3 bed vol. Binding Buffer until the absorbance at 280 nm reached the baseline, then washed with Wash Buffer (50 mM potassium phosphate pH 7.6, 0.5 M NaCl, 50 mM imidazole, 1 mM EDTA and 0.01% α-mercaptoethanol) until the absorbance at 280 nm reached the baseline. The enzyme was eluted Elute Buffer (50 mM potassium phosphate pH 7.6, 0.5 M NaCl, 300 mM imidazole, 1 mM EDTA and 1 mM DTT). Active fractions were pooled, and dialyzed against 50 vol of 20 mM potassium phosphate pH 7.6 containing 1 mM DTT and 1 mM EDTA. The final product with 30% glycerol and 1mM NAD was stored at -80° C.

The recombinant SAHase was purified by a two-step procedure, ammonium sulfate and affinity chromatography, which is particularly fast and efficient. The purified preparation gave a single band by SDS-polyacrylamide gel electrophoresis. The specific activity of purified rSAHase is 1.79 units/mg protein according to the above method.